Influenza viruses are major pathogens accountable for infectious, acute respiratory illness in the human population around the world. The accurate and well-timed diagnosis of influenza viruses is useful for early clinical management as well as monitoring and controlling the disease outbreak at initial stages. The present study was designed for rapid, sensitive, specific and cost-effective detection and identification of influenza viruses using RT-LAMP assays. Typing and subtyping RT-LAMP assays were developed for diagnosis of influenza viruses. Typing RT-LAMP assays were optimized for detection of influenza A and B viruses whereas subtyping RT-LAMP assays were standardized for detection of influenza A virus subtypes. RT-LAMP assays were evaluated on clinical specimens and the results were compared with conventional RT-PCR, real-time RT-PCR and virus isolation. Sequencing and phylogenetic studies were also performed on some of influenza A isolates. The major outcomes of the research work have been summarized below.

Typing of influenza viruses was done by using published primers, targeting matrix (M) gene and nucleoprotein (NP) gene of influenza A and B virus, respectively. Similarly, for subtyping of influenza A viruses into A(H1N1)pdm09, H3N2 and H1N1, primers were taken from hemagglutinin (HA) gene. All the RT-LAMP reactions demonstrated good amplification of the respective target template when incubated at 63°C for 1 h after which the reactions were terminated by incubating at 80°C for 5 min. RT-LAMP amplicons were detected by using agarose gel electrophoresis and by visual detection of green fluorescence of added SYBR green dye. The positive amplicons showed ladder-like pattern in gel electrophoresis and presence of green fluorescence during visual detection under UV/blue light. RT-LAMP assays were highly specific for detection of their respective influenza strains and no cross-reactive amplifications were observed between subtypes of influenza viruses as well as with other respiratory viruses.

RT-LAMP assays were compared for analytical sensitivity with conventional RT-PCR as well as with WHO recommended TaqMan rRT-PCR. Monoplex typing and subtyping conventional RT-PCR reactions were optimized in separate reactions using extracted RNA from the respective reference strain. Published primer pairs were selected from M gene and NP gene for typing of influenza A and B viruses, respectively.
Subtyping of IAVs was done by using published primer sets from HA gene. Monoplex typing and subtyping conventional RT-PCR reactions were later combined in two separate multiplex formats. Both typing and subtyping multiplex conventional RT-PCRs showed 100% correlation with the results of monoplex RT-PCRs.

The detection limits of typing RT-LAMP assays was 0.01 PFU/reaction for influenza A and 1 PFU/reaction for influenza B viruses. Subtyping RT-LAMP assays demonstrated detection limits of 0.01, 0.1 and 0.1 PFU/reaction for detecting A(H1N1)pdm09, H3N2 and H1N1, respectively. Analytical sensitivities of RT-LAMP assays were comparable to that of TaqMan rRT-PCR and 10 to 100-fold higher than conventional RT-PCR for detecting types and subtypes of influenza viruses. Following RNA extraction, time of detection for RT-LAMP reaction was <2 h making it faster diagnosis method than conventional RT-PCR (4 h) and TaqMan rRT-PCR (3 h).

A total of 252 nasal/throat swab samples were collected from patients with suspected influenza infections during September 2014 to August 2017 from PGIMS, Rohtak, Haryana, India. Maximum numbers of suspected influenza cases (28%) were from patients between the age group 21-30 years. The clinical samples were processed for virus isolation using MDCK cells. HI tests were done for typing and subtyping of influenza positive clinical isolates. Virus isolation detected 35/252 (13.8%) samples as influenza positive which were further typed as 31/35 (88.6%) influenza A and 4/35 (11.4%) as influenza B. Subtyping of influenza A isolates detected 23/31 (74.2%) positive for A(H1N1)pdm09 and 8/31 (25.8%) positive for H3N2 subtype. No clinical specimen was found positive for H1N1 subtype by virus isolation. Among clinical symptoms of influenza positive cases, fever ($P = 0.008$ Fisher’s exact) and cough ($P = 0.018$ Fisher’s exact) were found significantly related with the frequency of influenza infections.

TaqMan rRT-PCR was performed on clinical samples for detection and identification of influenza viruses by following WHO protocol. A total of 61/252 (24.2%) samples were detected positive for influenza viruses which were further typed into 55 (90.2%) influenza A and 6 (9.8%) influenza B type. Subtyping TaqMan rRT-PCR marked 37/55 (67.3%) samples as A(H1N1)pdm09, 14/55 (25.4%) as H3N2 and 4/55 (7.3%) as
H1N1 subtype. TaqMan rRT-PCR was 100% sensitive compared to virus isolation for detection, typing and subtyping of influenza viruses.

Clinical samples were also evaluated for the presence of influenza viruses by RT-LAMP and conventional multiplex RT-PCR. A total of 57/252 (22.6%) samples were tested positive by RT-LAMP assays whereas by conventional multiplex RT-PCR 49/252 (19.4%) samples were positive. Typing RT-LAMP assays detected 51/57 (89.5%) samples of influenza A and 6/57 (10.5%) of influenza B type. Influenza A positive specimens were further differentiated by subtyping RT-LAMP assays into 33 (64.7%), 14 (27.5%) and 4 (7.8%) as A(H1N1)pdm09, H3N2 and H1N1, respectively. Applying conventional multiplex RT-PCR, 44/49 (89.8%) samples typed as influenza A and 5/49 (10.2%) typed as influenza B. Subtyping conventional multiplex RT-PCR identified 31/44 (70.5%), 11/44 (25%) and 2/44 (4.5%) samples positive for A(H1N1)pdm09, H3N2 and H1N1, respectively. RT-LAMP assays performed better than conventional RT-PCR and virus isolation during clinical specimen evaluation.

Comparative analysis RT-LAMP assays with virus isolation displayed sensitivity, specificity, PPV and NPV of 100%, 89.86%, 61.4% and 100%, respectively for detection of influenza viruses in clinical specimens. For influenza subtype A(H1N1)pdm09 diagnosis, RT-LAMP assays showed sensitivity, specificity, PPV and NPV of 100%, 95.63%, 69.7% and 100%, respectively compared to virus isolation. All positive specimens by virus isolation were also identified as positive with RT-LAMP assays. WHO’s TaqMan rRT-PCR exhibited maximum influenza positive detection from clinical specimens. For detection of influenza viruses, RT-LAMP assays demonstrated sensitivity, specificity, PPV and NPV of 93.44%, 100%, 100% and 97.95%, respectively compared to TaqMan rRT-PCR. Subtyping RT-LAMP assays displayed sensitivity, specificity, PPV and NPV of 89.19%, 100%, 100% and 98.17%, respectively for detection of A(H1N1)pdm09 subtype. RT-LAMP assays were found more sensitive and specific than conventional RT-PCR and virus isolation with reference to TaqMan rRT-PCR.

Partial HA gene sequences of six influenza A(H1N1)pdm09 isolates were analysed with respect to the reference strain A/California/7/2009. Sequence analyses of all six isolates showed the presence of conserved pattern in the amino acid substitutions of P100S, D114N, K180Q, S202T and S220T in the HA1 domain. All isolates
demonstrated 98-99% similarity between HA gene sequences among themselves and 97-98% similarity with the reference strain. Phylogenetic analysis revealed that all six influenza A(H1N1)pdm09 isolates were clustered together with other representative strains of clade 6B. Partial HA gene sequence of four H3N2 isolates were analysed for amino acid substitutions with reference to reference strain A/Texas/50/2012. Two conserved amino acid substitutions of N161S and P214S were observed along with variable changes in amino acid sequences of the four H3N2 isolates as compared to the reference strain. All H3N2 isolates revealed 97-98% nucleotide homology with the reference strain and 97-99% homology among themselves. Phylogenetic analysis of partial HA gene sequences of H3N2 isolates revealed the presence of two subgroups. Two isolates were grouped in a same clade with global strains including A/West Virginia/08/2016 and A/Maryland/20/2016. The other two isolates were clustered in other clade along with strains like A/India/M09/2013 and A/Florida/38/2014. All four H3N2 isolates were grouped in two different genogroups and were drifted from the A/Texas/50/2012-like lineage.

On the basis of the results of the present study, it is concluded that RT-LAMP assays performed well for detection, typing and subtyping of influenza viruses. RT-LAMP assays displayed high analytical as well as clinical sensitivities than conventional RT-PCR. RT-LAMP assays revealed high specificity during clinical evaluation as compared to virus isolation. These assays were accurate, fast and easy to perform and showed comparable sensitivity and specificity with high-end diagnostic tools like TaqMan real-time RT-PCR without the need of costly equipments. Typing and subtyping RT-LAMP assays can be used as valuable tools for early patient diagnosis during influenza outbreaks. Influenza viruses are highly variable and continuous surveillance of the circulating strains is required for developing effective vaccination strategies and assessment of antiviral resistance among emerging strains. Fast, reliable and cost-effective methods such as RT-LAMP can be used efficiently for influenza diagnosis, monitoring and surveillance studies especially in the resource-limited laboratory settings of the developing countries.